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Enhancement of Dendritic Cell-Based Immunotherapy Using a Small Molecule TGF-beta Receptor Type I Kinase Inhibitor

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14. ABSTRACT Dendritic cells (DC) have become particularly attractive candidates for cancer immunotherapy due to their potent ability to stimulate antigen specific T cells responses. To date DC-based immunotherapy has demonstrated only limited clinical success in the treatment of established tumors. The limited clinical efficacy of existing DC-based cancer vaccines has been attributed in part to suppressive factors produced by the growing tumor, such as transforming growth factor-beta (TGF-β) that has been shown to impair the immunostimulatory capacity of DCs. Therefore, strategies to neutralize the deleterious effects of TGF-β may lead to more effective DC-based cancer therapies. SM16 is a potent small molecule TGF-β receptor type I (TβRI) kinase inhibitor that binds to the ATP-binding pocket of this receptor. Here we show that SM16 inhibits the growth and metastasis of established 4T1 mammary tumors when delivered either via daily i.p. injection or orally through rodent chow. Our data indicate that the anti-tumor effects of oral SM16 were superior to those induced by i.p. injection. In addition, we demonstrate that the anti-tumor efficacy of SM16 is dependent on cellular immunity. DC vaccination failed to improve the efficacy of i.p. SM16, but DC vaccines combined with orally administered SM16 increased primary tumor regression. Furthermore, DC+SM16 therapy enhanced T-cell infiltration into the primary tumor and splenocytes isolated from mice on the combination therapy displayed enhanced IFN-γ production and anti-tumor CTL activity.

15. SUBJECT TERMS
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Key Accomplishments

Year 1:

1. HTS466284 inhibits Smad2 phosphorylation in primary 4T1 tumors *in vivo*.
2. HTS466284 does not consistently improve the efficacy of DC vaccines in the treatment of primary and metastatic 4T1 tumors.
3. HTS466284 does not augment the ability of DC vaccines to treat residual 4T1 metastatic disease.
4. SM16 suppresses Smad2 phosphorylation in 4T1 tumor cells *in vitro* and in primary and metastatic 4T1 tumors *in vivo*.
5. SM16 inhibits TGF-β-induced 4T1 tumor cell invasion *in vitro.*
6. SM16 inhibits the growth of pulmonary 4T1 metastases *in vivo* at a dose of 40mg/kg.

Year 2:

1. 40mg/kg i.p. SM16 inhibits the growth rate of 7-day established 4T1 tumors.
2. DC vaccination does not improve the efficacy of i.p. SM16 in the treatment of 4T1 tumors.
3. Orally delivered SM16 inhibits Smad2 phosphorylation in established primary 4T1 tumors.
4. Oral administration of SM16 inhibits primary and metastatic 4T1 tumor growth.
5. Oral SM16 has no effect on primary and metastatic 4T1 tumors in immunodeficient mice.
6. Primary tumor regression is enhanced in animals treated with oral SM16 in combination with DC vaccination compared to oral SM16 alone.
7. The combination of DC and oral SM16 induces enhanced anti-tumor immunity.

For a detailed description of key accomplishment refer to the results section.

Reportable Outcomes

Publications:

1. **Rausch MP**, Hahn T, Ramanathapuram L, Bradley-Dunlop D, Mahadevan D, Mercado-Pimentel ME, Runyan RB, Besselsen DG, Zhang, X, Cheung HK, Lee WC, Ling LE, Akporiaye ET. An orally active small molecule TGF-β receptor I antagonist inhibits the growth of and metastatic murine breast cancer. *Anticancer Research*. (Accepted October 2008)

Presentations:

1. **Rausch MP**, Mahadevan D, Lee, WC, Zhang, X, Cheung HK, Ling LE, Akporiaye ET.Disruption of TGF-β signaling using a small molecule TGF-βRI antagonist improves the efficacy of dendritic cell vaccines for breast cancer. 5th Era of Hope Meeting, Baltimore, MD, June 25-28, 2008.
2. **Rausch, MP**, Mahadevan, D, Hahn, T, Ramanathapuram, L, Bradley-Dunlop, D, Zhang, X, Ling, L, Akporiaye, ET. Using a small molecule ALK5 antagonist to improve the efficacy of dendritic cell vaccines for breast cancer. 99th Annual Meeting of the American Association for Cancer Research, San Diego, CA, April 12-16, 2008.
3. **Rausch, MP**, Mahadevan, D, Zhang X, Ling, L, Akporiaye, ET. Disruption of tumor-derived TGF-β signaling using a small molecule ALK5 antagonist improves anti-tumor immunity. 3rd Annual Frontiers in Immunobiology & Immunopathogenesis Symposium, Tucson, AZ, March 1, 2008.

4. **Rausch, MP**, Mahadevan, D, Zhang X, Ling, L, Akporiaye, ET. Disruption of TGF-β signaling using small molecule TGFβRI antagonists improves the efficacy of dendritic cell vaccines for breast cancer. 2007 Graduate and Professional student Showcase, Tucson, AZ, Nov. 2-3, 2007.
5. **Rausch MP**, Mahadevan D, Zhang X, Ling L, Akporiaye ET. Disruption of TGF-β signaling using small molecule TGFβRI antagonists improves the efficacy of dendritic cell vaccines. 2nd Annual Frontiers in Immunobiology & Immunopathogenesis Symposium, Tucson, AZ, April 21, 2007.
6. **Rausch MP**, Mahadevan D, Akporiaye ET. Enhancement of dendritic cell-based immunotherapy for breast cancer using a small molecule TGF-β kinase inhibitor. 2006 Graduate and Professional Student Showcase, Tucson, AZ, Nov. 10-11, 2006.

Training Accomplishments:

Over the course of the two and a half years of this award, I was able to present my work at several national meetings including the 99th Annual Meeting of the American Association for Cancer Research and the 5th Era of Hope Meeting. In addition, I also presented posters on this work at several local meetings. These opportunities led to several important collaborations that both strengthened the impact of my work and introduced me to a number of new techniques. One of these collaborations eventually developed into a postdoctoral position in Karen Hastings' lab at the University of Arizona College of Medicine Phoenix where I will continue my training in cancer research studying antigen presentation and the immune response to melanoma.

On December 10th, 2008 I successfully defended my dissertation, completing the requirements for my Ph.D. in Microbiology and Immunology. A publication on the work funded by this grant entitled "An orally active small molecule TGF-β receptor I antagonist inhibits the growth of and metastatic murine breast cancer" (see attached .pdf file) was accepted by Anticancer Research in October 2008 and a second publication focused on the DC-based immunotherapy aspects of this project is currently in preparation for submission this Fall.

INTRODUCTION

Dendritic cells (DC) have become particularly attractive candidates for cancer immunotherapy due to their potent ability to stimulate antigen specific T cells responses. A number of pre-clinical and clinical studies using tumor antigen-pulsed DCs to treat a variety of malignancies have demonstrated that DC vaccines can elicit measurable cellular anti-tumor immunity. However, despite these encouraging results, DC-based immunotherapy has demonstrated only marginal clinical success in the treatment of established tumors in cancer patients. These limitations provide rationale for investigating new strategies to augment the efficacy of existing DC-based cancer vaccines. The limited clinical efficacy of DC-based cancer vaccines has been attributed in part to suppressive factors produced by the growing tumor, such as transforming growth factor-beta (TGF-β). Therefore, strategies to neutralize the deleterious effects of TGF-β may lead to more effective DC-based cancer therapies. Small molecule TGF-β signaling antagonists that block TGF-β signaling by selectively inhibiting the kinase activity of the TGF-β receptor type I (TβRI/ALK5) represent one promising approach to block tumor-derived TGF-β-mediated immunosuppression (1-2). The goal of this study is to use small molecule ALK5 kinase inhibitors to enhance the effectiveness of DC-based cancer vaccines in the treatment of established and metastatic TGF-β-producing murine mammary tumors. The hypothesis to be tested is that small molecule ALK5 kinase inhibitor therapy will enhance the efficacy of DC vaccines in the treatment of murine mammary tumors by rendering DCs resistant to TGF-β-mediated immunosuppression. The specific aims of this study are to: 1) Determine the effect of ALK5 kinase inhibitors on spontaneous tumor metastasis, 2) Evaluate the effect of the combination ALK5 kinase inhibitors plus DC vaccination on the treatment of primary and metastatic breast cancer, 3) Evaluate the role of immune effector cells in the anti-tumor response following combination therapy with ALK5 kinase inhibiotrs and DC vaccines.

RESULTS

Year 1 (July 1st 2006 - June 30th 2007):

<u>Aim #1: Evaluate the ability of HTS466284 to suppress the formation of spontaneous tumor metastasis</u>

HTS466284 therapy has no effect on the growth and metastasis of established 4T1 mammary tumors

To determine the effect of HTS466284 on the growth and metastasis of established 4T1 mammary tumors mice bearing 12-day established 4T1 tumors were treated with either 10, 45, or 60 mg/kg body weight of HTS466284 via daily *i.p.* injection. Primary tumor size (mm^2) was measured weekly and pulmonary metastases were enumerated at the time of sacrifice. The data indicate that HTS466284 at doses of 10 or 45 mg/kg had no effect on the growth of established primary 4T1 tumors (Figure 1A and C). Furthermore, at these doses HTS466284 had no effect on pulmonary metastasis formation in these animals (Figure 1B and D). HTS466284 was well tolerated and animals in these treatment groups displayed no overt signs of toxicity. However, HTS466284 at a dose of 60 mg/kg was toxic with more than 50% of the mice dying after only two injections forcing us to terminate the study.

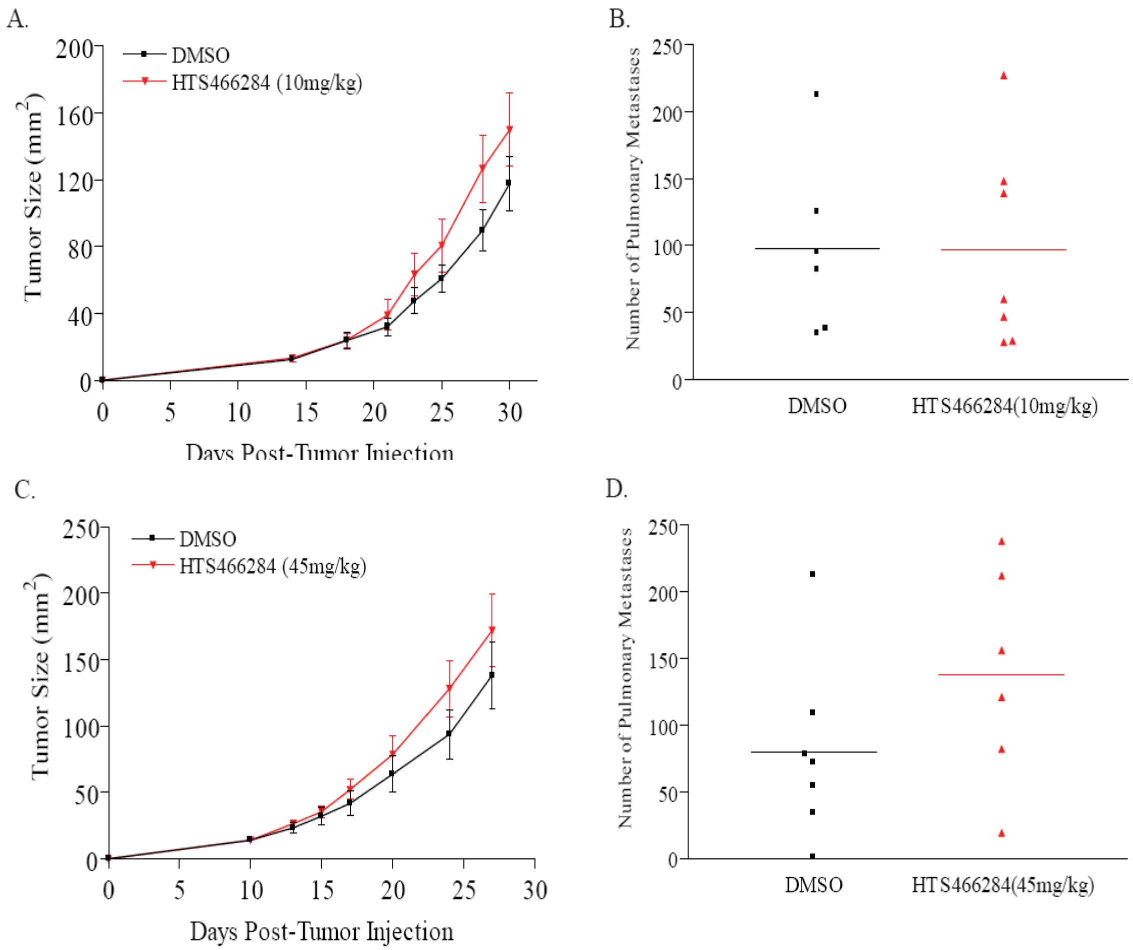


Figure 1. HTS466284 therapy alone had no effect on the growth and metastasis of established 4T1 tumors. Six-week-old female BALB/c mice bearing established 4T1 tumors were treated with 10 (**A and B**) or 45 (**C and D**) mg/kg of HTS466284 by daily *i.p.* injection for 17 days. Control animals received 17 daily injections of DMSO alone. The data represent: **A and C.** Mean tumor size (mm^2) ± SEM of 7 individual mice and **B and D.** Number of pulmonary metastases at the time of sacrifice on day 28.

Aim #2: Evaluate the effect of the combination of HTS466284 and DC vaccination on the treatment of primary and metastatic breast *in vivo*

HTS466284 in combination with autologous DC vaccination fails to consistently inhibit primary tumor growth and metastasis

Previous work in our laboratory has shown that tumor-derived TGF-β suppresses the effectiveness of DC vaccines in the treatment of 4T1 mammary tumors (6). The ability of HTS466284 to restore the immunostimulatory capacity of TGF-β treated DCs *in vitro* (preliminary data) suggested that this drug may be an effective strategy to improve efficacy of DC vaccines in the treatment of TGF-

β-producing tumors. Therefore, we hypothesized that HTS466284 would augment the efficacy of DC-based vaccines in the treatment of established 4T1 mammary tumors. To test this hypothesis, mice bearing established 4T1 tumors were treated with a non-toxic dose of HTS466284 (10 mg/kg body weight) in combination with 4T1 tumor lysate-pulsed, TNF-α-matured DCs. The data indicate that treatment with HTS466284 and DC vaccination had no effect on the growth of established primary tumors (Figure 2A). In addition, combination therapy had no effect on the formation of spontaneous pulmonary 4T1 metastases (Figure 2B).

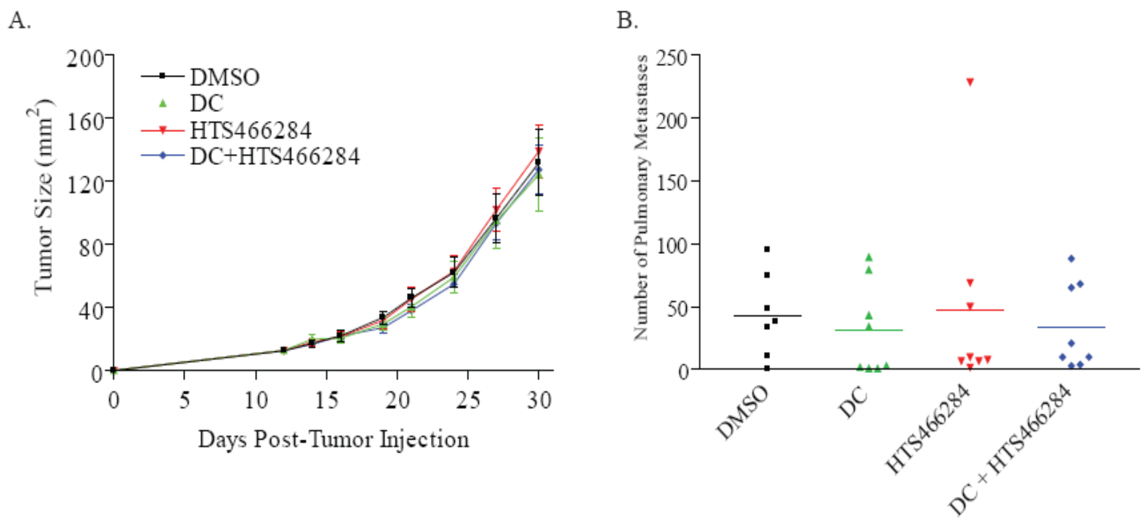


Figure 2. Combination therapy with HTS466284 (10mg/kg) and DC vaccination had no effect on the growth and metastasis of established 4T1 tumors. Six-week-old female BALB/c mice bearing 10-day established 4T1 tumors were treated with 10mg/kg of HTS466284 by daily *i.p.* injection for 17 days. Control animals received 17 daily injections of DMSO alone. Mice in the DC groups were injected s.c. contralateral to the tumor site with $2x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 12, 17, and 22. The data represent: **A.** Mean tumor size (mm^2) ± SEM of 7 individual mice and **B.** Number of pulmonary metastases at the time of sacrifice on day 30.

Since HTS466284 at a dose of 10 mg/kg body weight in combination with DC vaccination had no effect on established 4T1 tumors, we increased the dosage of HTS466284 to 45 mg/kg body weight (the maximum tolerated dose from our preliminary studies). As described above, mice bearing established 4T1 tumors were treated with HTS466284 (45 mg/kg) in combination with 4T1 tumor lysate-pulsed, TNF-α-matured DCs. As seen in Figure 3A, the combination of DC+HTS466284 significantly inhibited primary tumor growth compared to control treatments. Tumors from mice in the combination therapy group grew at a significantly slower rate than tumors from animals treated with DMSO alone ($p<0.05$), HTS466284 alone ($p<0.01$), or DC alone ($p<0.05$) (Figure 3A). The mean tumor volumes on day 28 post-tumor injection in mice treated with DMSO, HTS466284, and DC alone were 162.6±35.2 mm^2, 163.5±33.5 mm^2, and 174.7±35.9 mm^2 respectively. In contrast, the mean tumor volume in mice receiving DC+HTS466284 on day 28 was 77.6±22.5 mm^2. In addition, the combination treatment regimen significantly prolonged survival ($p<0.05$) in these animals compared to mice treated with DMSO alone, HTS466284 alone, and DC alone (Figure 3B). The median survival time was increased from 30.5 days in animals treated with HTS466284 alone and 33 days in animals receiving DMSO or DC alone to 47 days in mice receiving the combination therapy. All of the mice treated with DMSO or HTS466284 alone died

from large tumor burden by day 39 and all animals treated with DC alone died by day 44. Remarkably, one mouse in the DC+HTS466284 combination therapy group survived until day 63.

We conducted a follow-up experiment to confirm the data from the previous experiment. To this end, mice bearing 12-day established 4T1 tumors were treated with 45 mg/kg of HTS466284 by daily *i.p.* injection and 4T1 tumor lysate-pulsed, TNF-α matured DCs as described above. Primary tumor growth was measured and pulmonary metastases were enumerated visually at the time of sacrifice. In this study we were unable to replicate our earlier findings. The data show that unlike what was seen previously, DC+HTS466284 (45 mg/kg) had no statistically significant effect on primary 4T1 tumor growth (Figure 3C). Furthermore, combination DC+HTS466284 therapy failed to significantly suppress the formation of spontaneous pulmonary 4T1 metastases (Figure 3D).

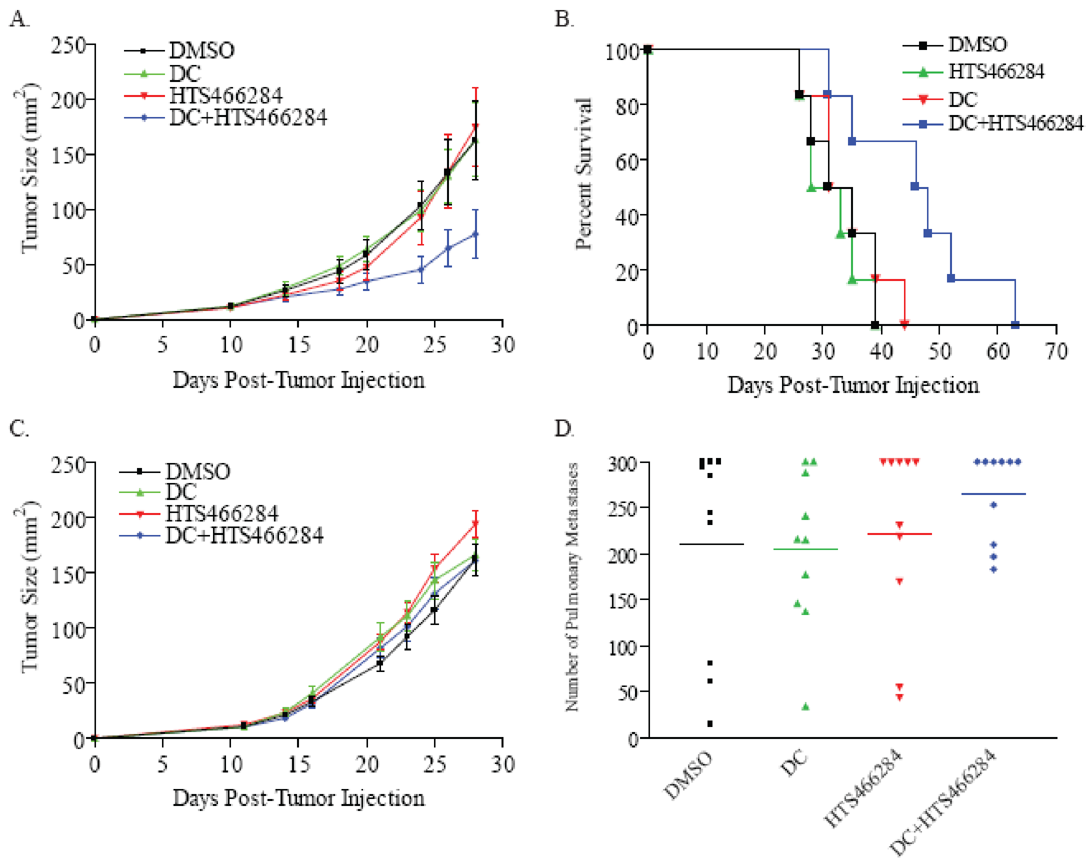


Figure 3. HTS466284 does not consistently improve the efficacy of DC vaccination for established 4T1 tumors. BALB/c mice bearing 12-day established 4T1 tumors were injected *i.p.* with 45mg/kg of HTS466284 in 50 µl of DMSO daily for 17 days. Control animals received DMSO. Mice in the DC groups were injected s.c. contralateral to the tumor site with $2x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 12, 17, and 22. The data represent: **A.** Mean tumor volume ± SEM of 7 individual mice in experiment #1, **B.** Percent survival of mice in each group. Animals either died naturally from tumor burden or were sacrificed when tumors reached an average size greater than 1500 mm^3 **C.** Mean tumor volume ± SEM of 10 individual mice in experiment #2, and **D.** Number of pulmonary metastases at the time of sacrifice on day 28.* indicates statistical difference ($p<0.05$) relative to control.

HTS466284 failed to enhance the ability of DC vaccines to treat 4T1 metastases in a residual disease setting.

Since most breast cancer related mortality is the result of disseminated disease, we wanted to evaluate if HTS466284 therapy can augment the ability of DC vaccination to treat residual metastatic disease. To this end, 4T1 tumors were established in Balb/c mice as described above. After 21 days when the tumors had reach an average of 97 mm^3, the tumors were surgically resected. The following day (day 22 post-tumor injection) the mice began receiving daily i.p. injections of 45mg/kg of HTS466284 and injections were continued until day 29 post-tumor injection. The mice were also injected s.c. with 10^6 4T1 tumor lysate-pulsed, TNF-α matured DCs on days 23 and 28. On day 30 the lungs were collected, stained with India ink as described previously, and surface lung metastases were counted visually. The data show that combination HTS466284+DC therapy had no significant effect on the growth of residual 4T1 pulmonary metastases (Figure 4).

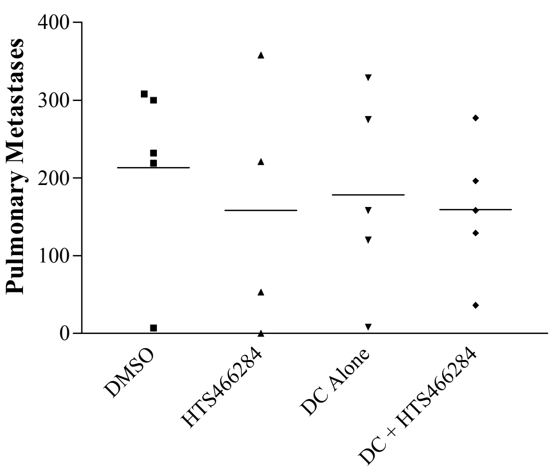


Figure 4 Effect of HTS466284 and DC vaccination on residual metastatic disease. Six-week-old female BALB/c mice were injected s.c. on day 0 with $5x10^4$ 4T1 cells into the mammary fat pad. On day 21 the tumors were surgically resected. The following day the mice were injected i.p. with 45 mg/kg of HTS466284 in 50 μl of DMSO daily for a total of 8 injections. Control animals received DMSO alone. Mice in the DC groups were injected s.c. with $1x10^6$ 4T1 tumor lysate pulsed, TNF-α matured DCs on days 23 and 28. On day 30 lungs were collected, perfused with India ink, fixed in Fekete's Solution, and surface lung metastases were counted visually. The data represent the number of pulmonary nodules from 5 individual mice.

<u>Aim #1: Evaluate the ability of SM16 to suppress the formation of spontaneous tumor metastasis</u>

SM16 inhibits TGF-β signaling in 4T1 tumor cells *in vitro* and 4T1 tumor tissue *in vivo*.

Since HTS466284 failed to control 4T1 tumor growth and since this drug also failed to improve the efficacy of DC vaccination in the treatment of 4T1 tumors in established and residual metastatic disease models, we shifted our focus to an improved small molecule ALK5 kinase inhibitor SM16. SM16 has demonstrated anti- tumor efficacy in a number of murine models (1) and in addition, has been shown to promote anti-tumor immunity in a murine model of mesothelioma (2). In order to assess the ability of SM16 to inhibit TGF-β signaling in the 4T1model, we assessed Smad2 phosphorylation (pSmad2) in cultured 4T1 tumor cells and 4T1 tumors from SM16-treated mice by western blot analysis. The data show that SM16 significantly blocked Smad2 phosphorylation in 4T1 tumor cells in a dose-dependent manner with near complete abrogation of pSmad2 occurring at a concentration of 5μM (Figure 5A). In addition, 5μM of SM16 completely inhibited pSmad2 in the absence of exogenous TGF-β, indicating that this drug is capable of blocking endogenous TGF-β signaling in 4T1 tumor cells (Figure 5A). We also evaluated the ability of SM16 to inhibit TGF-β signaling in established tumors. Mice bearing established 4T1 tumors were injected *i.p.* with a single dose of either 10 or 40 mg/kg body weight of SM16 and pSmad2

levels were analyzed in primary tumor and lung tissue bearing metastatic nodules. The data show that 10 or 40 mg/kg SM16 profoundly suppressed Smad2 phosphorylation in both primary tumors and lungs bearing a substantial number of metastatic nodules (Figure 5B). This effect was dose-dependent as inhibition of pSmad2 in the primary tumor was more effective at a dose of 40 mg/kg SM16 (Figure 5B).

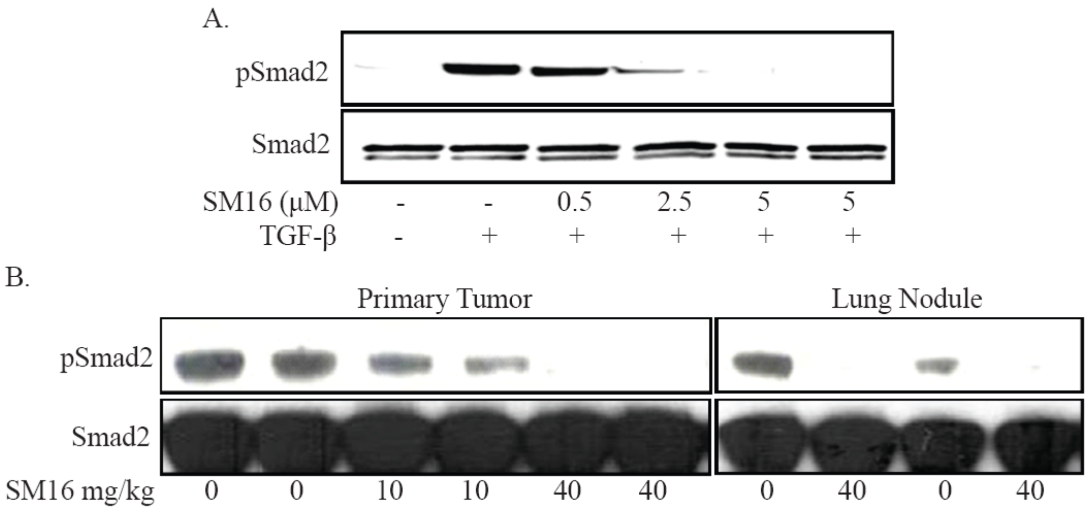


Figure 5. Effect of SM16 on TGF-β-induced Smad2 phosphorylation in cultured 4T1 tumor cells and 4T1 tumors. A. 4T1 cells were treated with SM16. Lysates were prepared and pSmad2 was detected by Western blot analysis **B.** Mice bearing established 4T1 tumors were injected *i.p.* with a single dose of SM16 (10 or 40mg/kg body weight) or vehicle (Captisol). One hr after treatment, primary tumors or lungs were homogenized and pSmad2 was detected by Western blot.

SM16 inhibits TGF-β-induced morphological changes and invasion of 4T1 cells

Previous work in our laboratory demonstrated that TGF-β plays an important role in migration and invasion of 4T1 tumor cells (3). In order to test the ability of SM16 to block TGF-β-mediated invasion, 4T1 cells were treated with the drug and assessed for their ability to invade Matrigel in response to exogenous TGF-β. As shown in Figure 6, TGF-β stimulated the ability of 4T1 cells to invade Matrigel nearly 3-fold. This effect was abrogated by treatment with 5μM SM16 ($p<0.001$) (Figure 6).

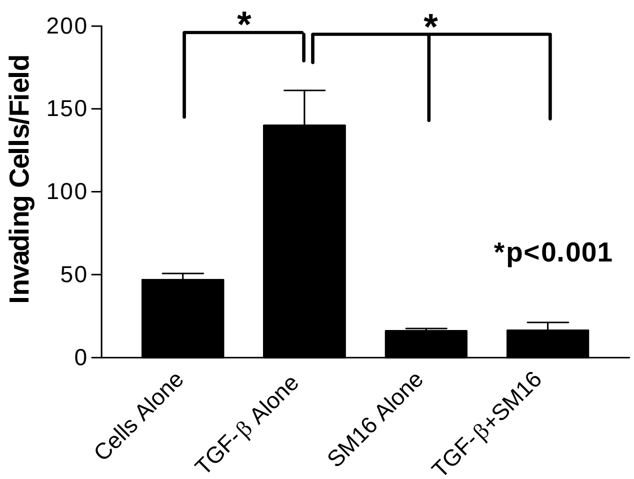
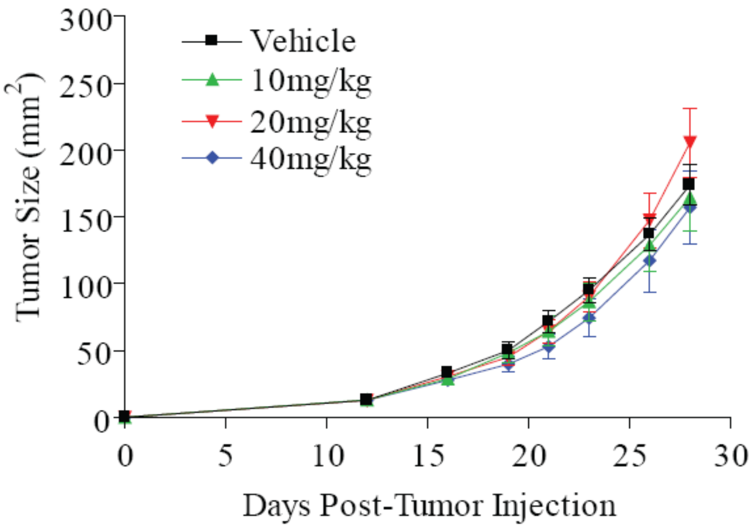


Figure 6. Effect of SM16 on 4T1 invasion *in vitro.* 4T1 cells were plated in growth factor-reduced Matrigel invasion cell culture inserts for 24 hr in the presence of 2 ng/ml TGF-β1 with or without 5μM SM16. Invading cells were stained with the DiffQuick staining kit and counted visually at 200X magnification. Data represent mean ± SEM of invading cells in 9 random fields of view. Data are representative of two independent experiments.

***i.p.* SM16 inhibits pulmonary metastasis formation**

In order to determine the optimal dose for SM16 therapy, mice bearing 10-day established 4T1 tumors were treated with various concentrations of SM16 via daily i.p. injection for 18 consecutive days. Control animals received 18 daily injections of 20% Captisol (vehicle). Primary tumor size was measured three times weekly and pulmonary metastatic burden was assessed at the end of the study. Figure 7A shows that SM16 had no significant effect on primary tumor growth at all concentrations tested. However, at a dose of 40 mg/kg, SM16 significantly inhibited the formation of pulmonary metastases compared to vehicle-treated controls ($p<0.05$) (Figure 7B). Animals treated with 40 mg/kg of SM16 had an average of 31±13 pulmonary metastatic nodules compared to an average of 121±23 nodules in vehicle-treated controls representing nearly a 4-fold reduction in the number of lung metastases. SM16 therapy was well tolerated at all doses and mice displayed no weight loss or other overt signs of drug-related toxicity. Based on these findings, the 40 mg/kg dose of SM16 was chosen for subsequent experiments.

A.



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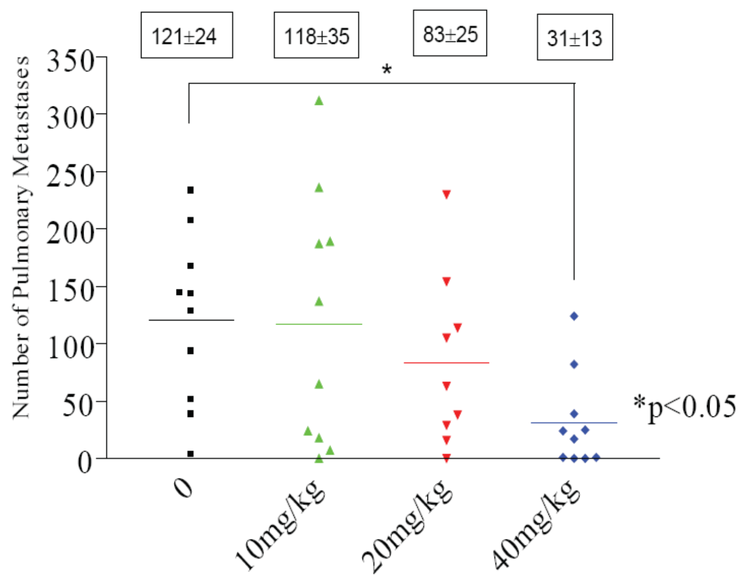


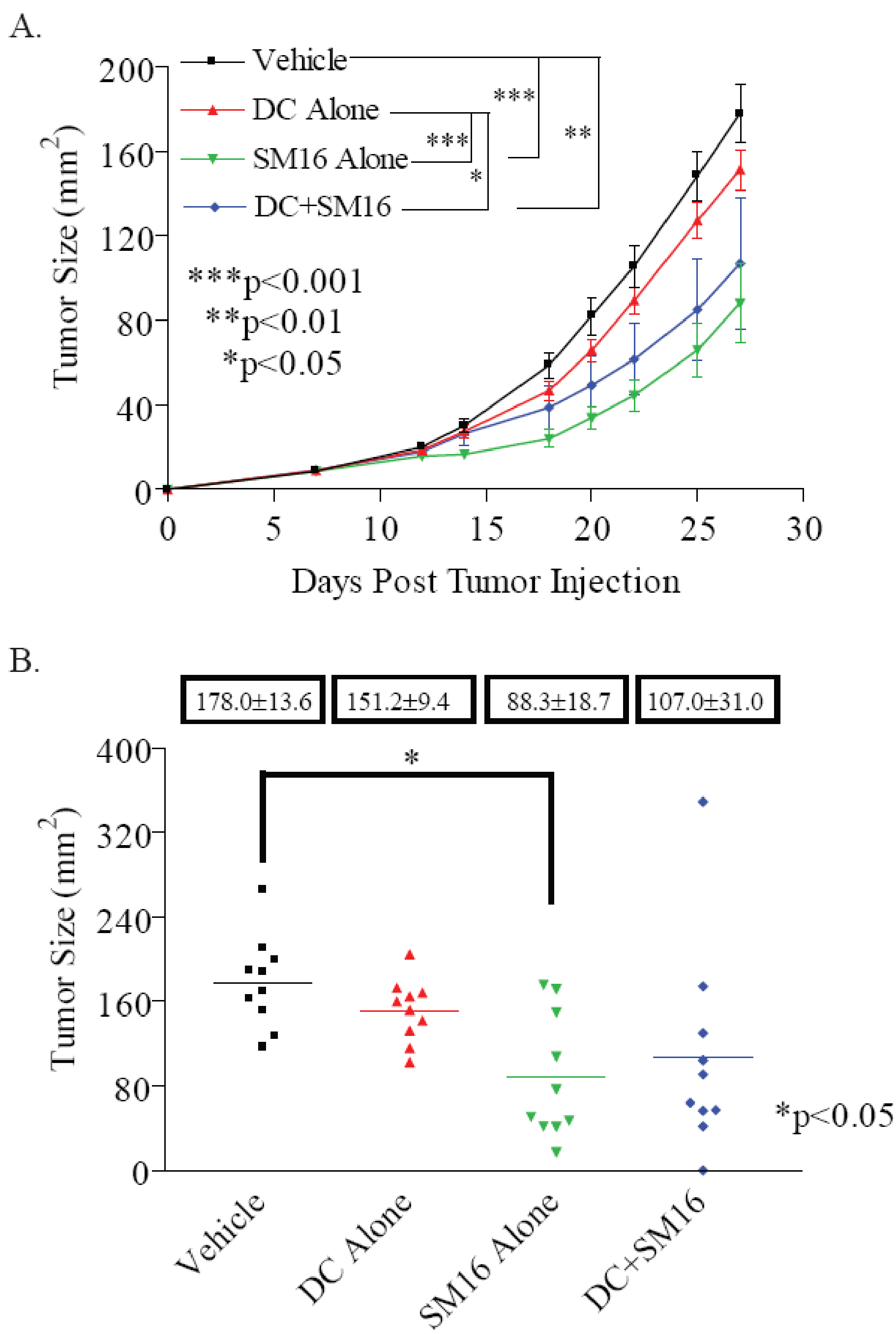
Figure 7. Comparison of various doses of SM16 (*i.p.*) on primary 4T1 tumor growth and metastasis formation. BALB/c mice bearing 12mm^2 4T1 tumors were treated *i.p.* with various doses of SM16. SM16 injections were continued daily until day 28. The data represent: **A.** Average tumor size (mm^2±SEM) and **B.** Number of pulmonary nodules at the time of sacrifice on day 28 (boxed numbers represent mean±SEM).

Year 2 (July 1st 2007 - June 30th 2008):

<u>Aim #2: Evaluate the effect of the combination of SM16 and DC vaccination on the treatment of primary and metastatic breast *in vivo*</u>

***i.p.* SM16 does not enhance the efficacy of DC vaccines**

The ability of tumor-derived TGF-β to impair anti-tumor immune responses has been well documented. Our laboratory has previously shown that inhibition of TGF-β signaling using the TGF-β neutralizing antibody 2G7 could enhance the efficacy of adoptively transferred DCs to treat 4T1 tumors (6). Furthermore, inhibition of ALK5 with SM16 has been shown to enhance endogenous anti-tumor immunity in a murine model of mesothelioma (2). Therefore, we wanted to determine if ALK5 kinase inhibition with SM16 could enhance the anti-tumor effect of DC vaccination. To this end, mice bearing (~9mm^2) established 4T1 tumors were treated with daily *i.p.* injections of SM16 (40mg/kg) in combination with 4T1 tumor lysate-pulsed, TNF-α-matured DC. The data indicate that tumors treated with *i.p.* SM16 alone at a dose of 40 mg/kg grew at a significantly slower rate that tumors treated with vehicle ($p<0.001$) or DC ($p<0.001$) alone (Figure 8A). Similarly, tumors from animals treated with the combination of DC+*i.p.* SM16 grew at a slower rate than tumors treated with vehicle ($p<0.01$) or DC alone ($p<0.05$) (Figure 8A). However, there was no significant difference in tumor growth between mice treated with *i.p.* SM16 alone and those treated with DC+*i.p.* SM16. At the time of sacrifice on day 28, tumors treated with *i.p.* SM16 or DC+*i.p.* SM16 had average sizes of 88.3±18.7 mm^2 or 107.0±31.0 mm^2 respectively compared to 178.0±13.6 mm^2 for mice treated with the vehicle and 151.2±9.4 mm^2 for mice treated with DC (Figure 8B). Interestingly, one of ten mice in the DC+*i.p.* SM16 group experienced complete tumor regression (Figure 8B). *i.p.* SM16 therapy alone or in combination with DC vaccination also significantly inhibited the formation of pulmonary metastases compared to controls (Figure 8C). Interestingly, both the *i.p.* SM16 alone and DC+*i.p.* SM16 groups had three animals with no pulmonary metastases (Figure 8C).


A.
Vehicle
DC Alone
SM16 Alone
DC+SM16
***
***
**
*
***p<0.001
**p<0.01
*p<0.05
Tumor Size (mm2)
200
160
120
80
40
0
0
5
10
15
20
25
30
Days Post Tumor Injection
B.
178.0±13.6
151.2±9.4
88.3±18.7
107.0±31.0
*
Tumor Size (mm2)
400
320
240
160
80
0
*p<0.05
Vehicle
DC Alone
SM16 Alone
DC+SM16

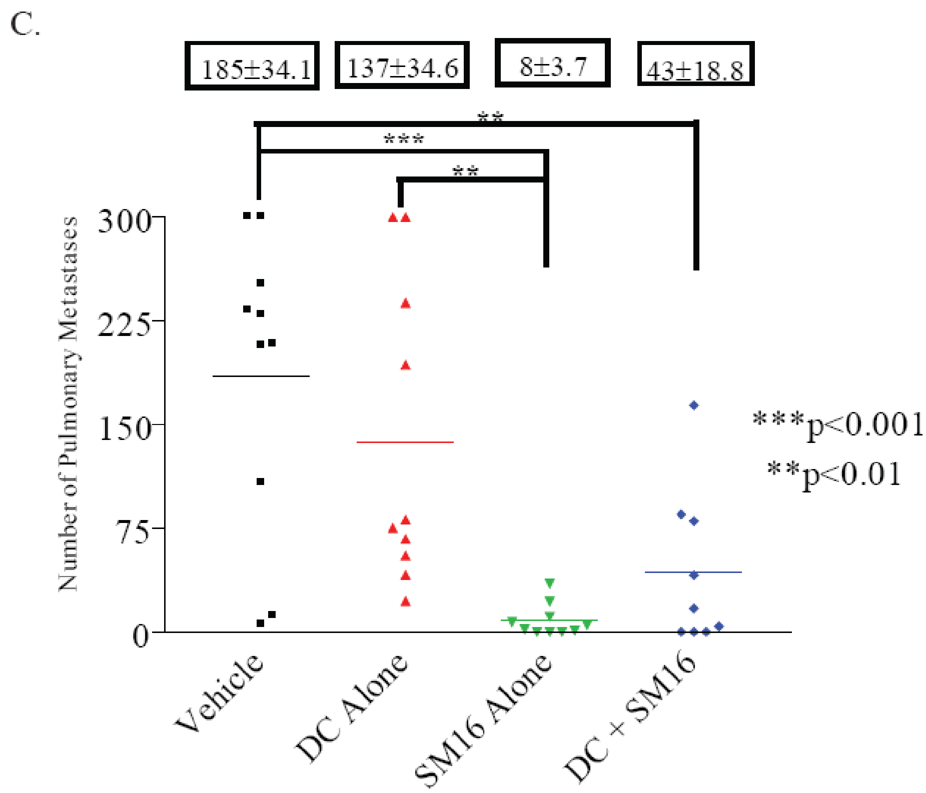


Figure 8. Effect of *i.p.* SM16 in combination with DC vaccination on the treatment of established 4T1 tumors. BALB/c mice bearing $9mm^2$ established 4T1 tumors were injected *i.p.* with 40mg/kg SM16. Injections were continued daily until day 28 for a total of 22 injections. Control animals received 20% Captisol (vehicle). Mice in the DC groups were injected s.c. with $1x10^6$ 4T1 lysate-pulsed, TNF-α-matured DC on days 12, 17, and 22 post tumor injection. The data represent: **A.** Average tumor size (mm^2) ± SEM **B.** Individual tumor sizes (mm^2) on day 28 **C.** Number of pulmonary nodules (boxed numbers represent mean±SEM).

Aim #1: Evaluate the ability of SM16 to suppress the formation of spontaneous tumor metastasis

Orally delivered SM16 inhibits Smad2 phosphorylation in established 4T1 tumors

The data from the DC+*i.p.* SM16 combination therapy study suggest that once daily *i.p.* injection may not be the most effective route of delivery for SM16. Therefore, we wanted to investigate alternative delivery strategies. A recent report by Suzuki et al. demonstrated that SM16 could be safely and effectively delivered orally through rodent chow to treat a murine model of mesothelioma (2). In addition, they found that the anti-tumor efficacy of oral (*p.o.*) dosing was superior to that of *i.p.* dosing (2). Therefore, we hypothesized that *p.o.* administration through rodent chow would allow us to achieve more continuous SM16 dosing than once daily *i.p.* injection since animals eat periodically over the course a day. This would produce more stable serum levels of the drug and ultimately result in more sustained and complete inhibition of TGF-β signaling. In order to test this hypothesis, SM16 was incorporated into standard mouse chow at a dose of 0.45 g

SM16/kg chow as described previously (2) and fed to mice bearing established 4T1 tumors in combination with DC vaccination as described above.

Before testing the anti-tumor efficacy of *p.o.* SM16, we wanted to determine if SM16 could block TGF-β signaling in the 4T1 tumor model when given orally. To this end, mice bearing established 4T1 tumors were fed SM16 diet for 24 hours before primary tumor tissue was analyzed for pSmad2 by western blot. The data show that tumors from animals on the SM16 diet had significantly reduced levels of phosphorylated Smad2 compared to tumors from animals on the control diet (Figure 9) clearly demonstrating that *p.o.* SM16 has the ability to inhibit TGF-β signal transduction in large 4T1 tumors.

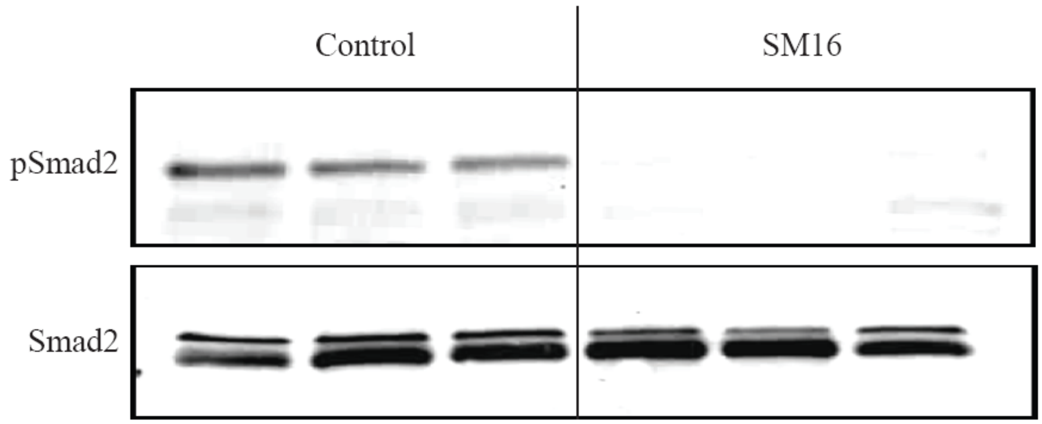


Figure 9. Effect of orally delivered SM16 on Smad2 phosphorylation in primary 4T1 tumors. Mice bearing established 4T1 tumors were fed either control diet or diet containing 0.45 g/kg food for 24 hr. Tumors were homogenized and pSmad2 and total Smad2 were detected by western blot analysis as described above.

SM16 diet is well tolerated and SM16 is detectable in serum

In order to determine the efficacy of orally delivered SM16, mice bearing established 4T1 tumors were fed SM16 diet and primary tumor growth was measured and lung metastases were enumerated. Each mouse consumed ~3.5g food per day which is equivalent to a daily dose of ~1.6mg or ~80mg/kg body weight per day. The animals showed no preference for either diet as total food consumption was similar in both groups (Figure 10A). In addition, animal weights were comparable in both groups and the SM16 diet was well tolerated with no overt signs of drug-related toxicity in the treated animals (Figure 10B). Analysis of serum from mice on the SM16 diet revealed detectable levels of the drug by 24 hours (Figure 10C). Serum SM16 levels increased slightly and reached a peak of 18.6±1.3μM after 36 hours. This peak level was maintained until the study was terminated on day 27 post- tumor injection (Figure 10C). As expected animals fed normal chow had no detectable levels of SM16 in their serum.

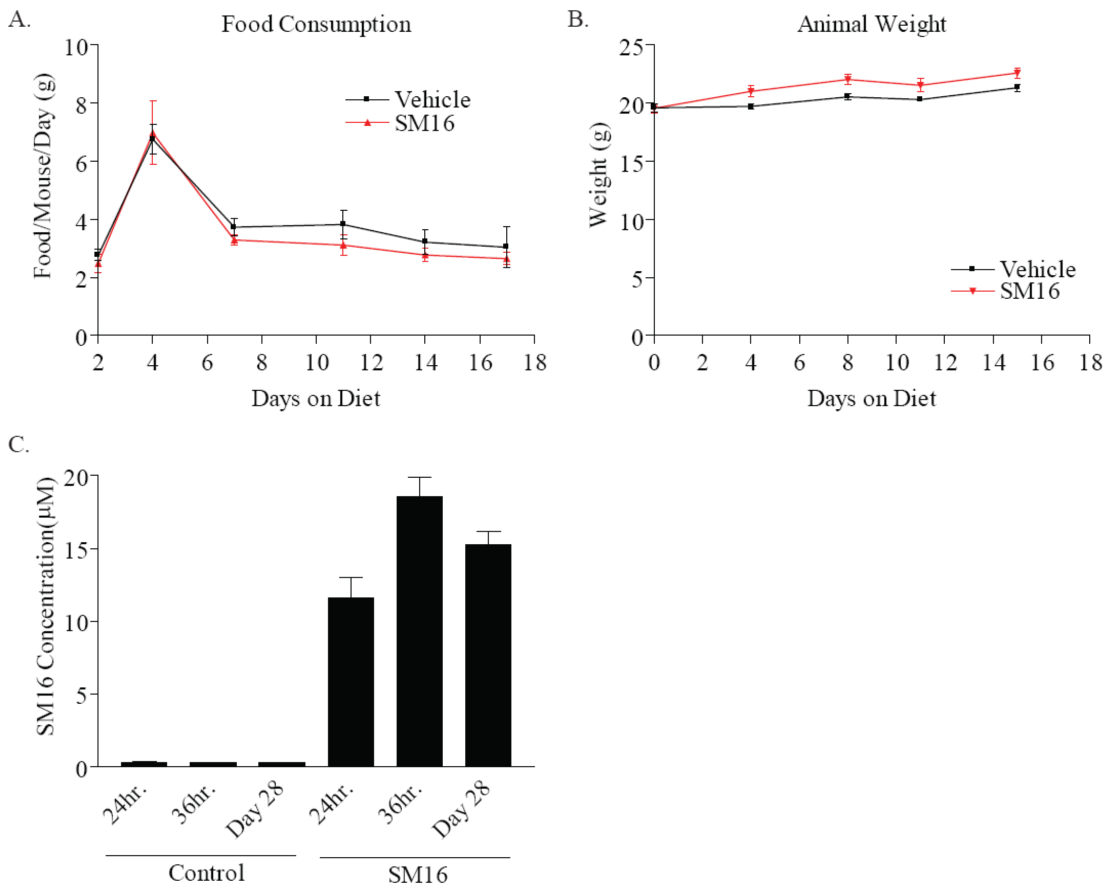


Figure 10. Analysis of SM16 food consumption and SM16 serum concentration. Mice bearing established 4T1 tumors were fed chow formulated with 0.45 g SM16/kg chow. Control animals received normal chow. Food and animals were weighed every 4 days. Serum was collected from control and SM16-fed animals after 24 hr, 36 hr, and 27 days on diet and analyzed for SM16 levels. The data represent: **A.** Average daily amount of food (g±SEM) consumed per mouse; **B.** Average weight of mice (g±SEM); **C.** Average SM16 serum levels (µM±SEM).

Oral administration of SM16 inhibits primary and metastatic tumor growth

Figure 11A shows that *p.o.* SM16 significantly inhibits the growth of established 4T1 tumors compared to animals receiving normal chow ($p<0.0001$). At the time of sacrifice on day 26, tumors from mice treated with *p.o.* SM16 had an average tumor size of 58.5±12.9 mm^2 versus 139.2±8.7 mm^2 for mice on normal chow, representing a 2.4 fold reduction in average tumor size ($p<0.0001$). One of ten mice in the SM16 diet group experienced complete regression of its primary tumor. Furthermore, the number of metastatic lung nodules was also significantly reduced in mice receiving the SM16 diet compared to mice receiving normal chow ($p<0.0001$) (Figure 11B). Taken together these data show that oral delivery of SM16 results in a significant suppression of primary tumor growth and metastasis formation that correlates with high serum levels of SM16 and inhibition of Smad2 phosphorylation within the primary tumor.

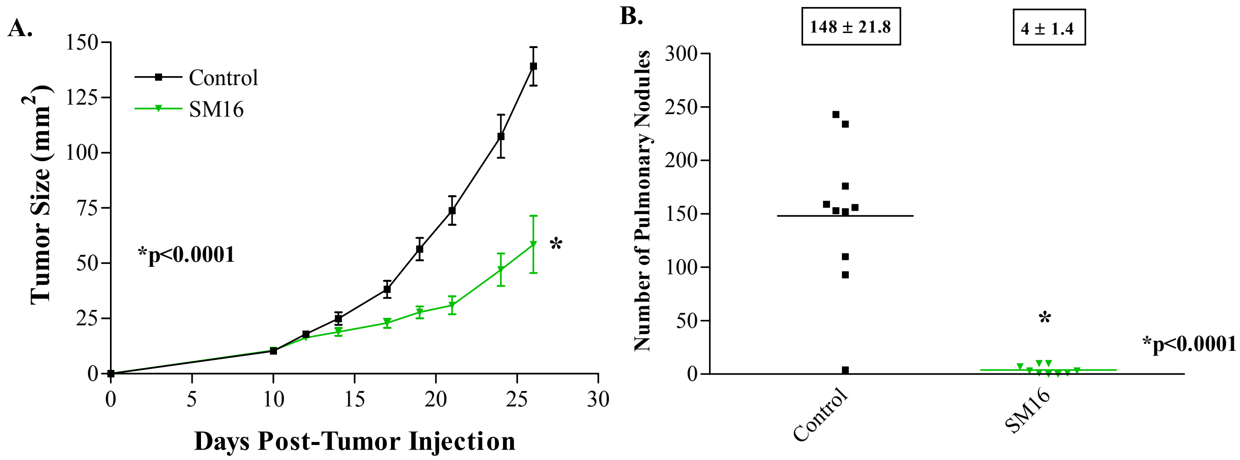


Figure 11. Effect of *p.o.* SM16 on primary tumor growth and metastasis formation. BALB/c mice bearing 10mm^2 4T1 tumors were transferred to chow formulated with 0.45g SM16/kg chow. Control animals received normal chow. Primary tumors were measured and pulmonary metastases were counted. The data represent: **A.** Average tumor size (mm^2±SEM); **B.** Number of pulmonary nodules at the time of sacrifice on day 27 (boxed numbers represent mean±SEM).

Oral SM16 has no effect on primary and metastatic 4T1 tumors in immunodeficient mice.

A number of recent reports have indicated that cellular immunity plays an important role in the anti-tumor efficacy of other small molecule ALK5 kinase inhibitors (2,4,5). Therefore, we wanted to determine if the anti-tumor effect of SM16 in the 4T1 tumor model was also immune mediated. For this purpose, SCID mice bearing 9-day established 4T1 tumors were fed either control or SM16 diet and primary tumor growth and pulmonary metastases were assessed. As shown in Figure 12A, there was no significant difference in primary tumor growth between SM16-fed and control animals. The tumors grew faster in these animals than in normal immunocompetent BALB/c mice and the study was terminated at day 21 post-tumor injection when animals became moribund. At this time, the average tumor sizes for SM16-fed and control animals were 174.9 ± 13.57 mm^2 and 135.1 ± 15.29 mm^2 respectively. Furthermore, there was no significant difference in the number of pulmonary metastases between animals on the SM16 and control diets (Figure 12B). Mice fed control chow had 259 ± 26.7 lung nodules compared to 287.0 ± 13.0 for mice fed SM16 chow. Taken together, these findings suggest that cellular immunity is critical for the anti-tumor and anti-metastatic activity of SM16.

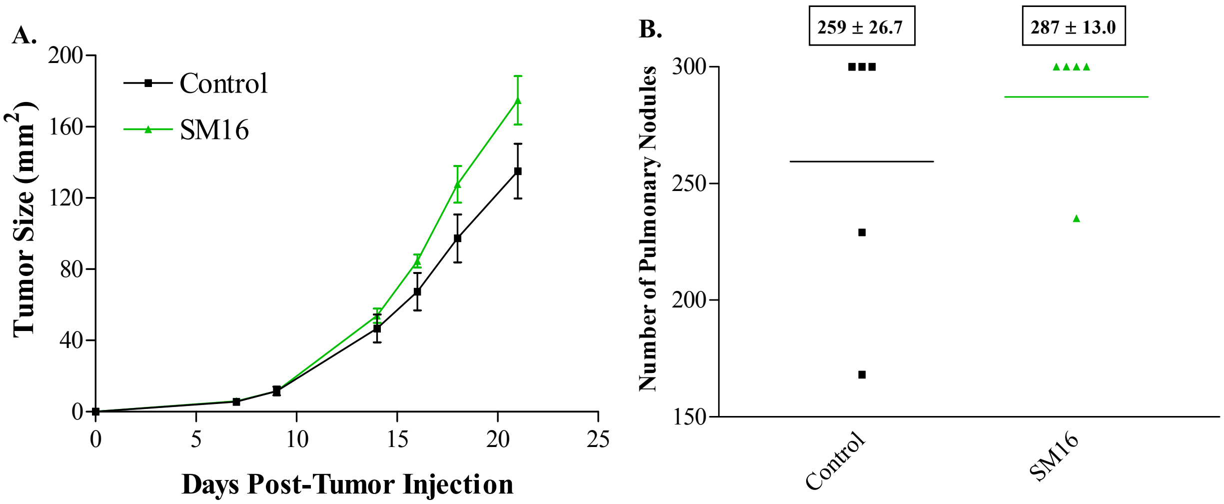


Figure 12. Effect of oral SM16 on 4T1 tumors in immunodeficient mice
Six-eight-week-old female C.B17/IcrACC*scid* mice were injected s.c. with 5x10^4 4T1 tumor cells into the mammary fat pad. On day 9 of tumor establishment, mice were fed diet containing 0.45g SM16/kg chow. Primary tumor sizes were measured and pulmonary metastases were enumerated visually. The data represent: **A.** Average tumor size (mm^2±SEM) and **B.** Number of pulmonary nodules (boxed numbers represent mean±SEM).

<u>Aim #2: Evaluate the effect of the combination of SM16 and DC vaccination on the treatment of primary and metastatic breast *in vivo*</u>

Inhibition of TGF-β signaling with oral SM16 enhances the ability of DC to induce primary tumor regression

Having shown that SM16 inhibits the growth of primary and metastatic 4T1 tumors through a mechanism involving cellular immunity, we wanted to determine if systemic TGF-β blockade with SM16 could enhance the anti-tumor activity of DC vaccines by blocking TGF-β-mediated immunosuppression. Therefore, mice bearing ~9-10mm^2 established tumors were vaccinated s.c. with 1x10^6 4T1 tumor lysate-pulsed, TNF-α matured BMDC with or without oral SM16 therapy. The data indicate that tumors from the SM16 alone and DC+SM16 groups grew at significantly slower rates ($p<0.01$) than controls (Figure 13A). Furthermore, the average tumor sizes in animals from the SM16 alone and DC+SM16 groups were significantly smaller ($p<0.05$) than those in the control groups (Figure 13B). At the time of sacrifice the average tumor sizes of the SM16 and DC+SM16 groups were 51.6±10.0 mm^2 and 64.7±25.7 mm^2 respectively compared to 128.3±10.1 mm^2 for the control diet group and 121.9±10.4 mm^2 for the DC alone group (Figure 13B). Interestingly, there was no significant difference in the primary tumor growth rate or average primary tumor size between the SM16 alone and DC+SM16 groups. Both of these groups appear to have responder and non-responder animals (Figure 14). Tumors from 6 of 12 animals in the SM16 alone group responded to therapy evidenced by either growth inhibition or complete regression (Figure 14). Similarly, 7 of 13 animals in the combination therapy group responded to treatment (Figure 14). However, the combination of DC+SM16 therapy induced complete primary tumor

regression in more responder animals than SM16 therapy alone (Figure 14 and Table 1). Five of seven responders (71.4%) treated with DC+SM16 underwent complete regression of their primary tumor compared to one of six animals (16.6%) treated with SM16 alone (Table 1). Animals treated with SM16 or DC+SM16 also had significantly fewer pulmonary metastases than control ($p<0.001$) or DC alone-treated animals ($p<0.01$) (Figure 20). The average number of metastatic nodules in the SM16 and DC+SM16 groups were 4±1.4 and 9±4.9 respectively compared to 148±21.8 for mice on the control diet and 97±27.7 for mice treated with DC alone (Figure 20).

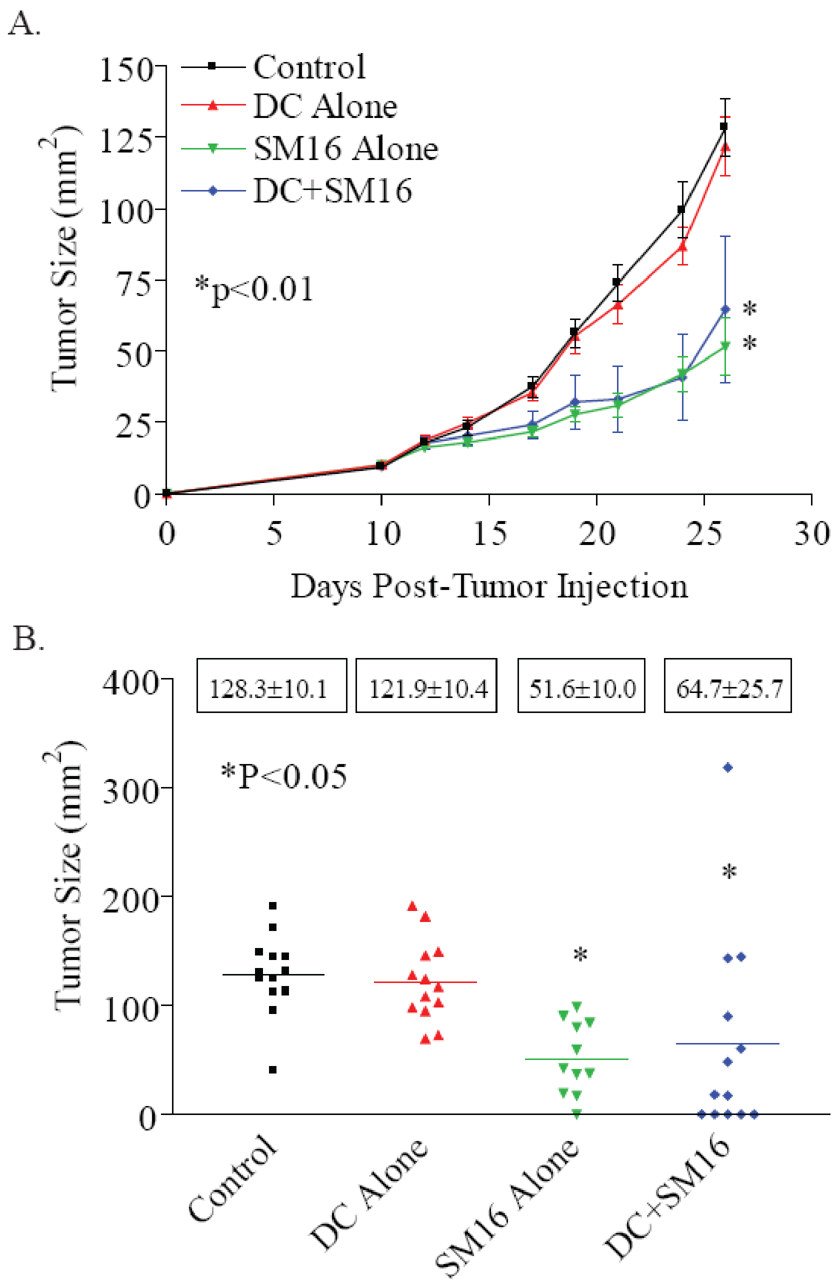


Figure 13. Effect of SM16 in combination with DC vaccination on established 4T1 tumors. BALB/c mice bearing ~9-10mm^2 established 4T1 tumors were transferred to chow formulated with 0.45g SM16/kg chow. Mice in the DC groups were injected with 1x10^6 4T1 lysate-pulsed, TNF-α

matured DCs on days 12, 17, and 22 post-tumor injection. Primary tumors were measured. The data represent: **A.** Average tumor size ($mm^2 \pm SEM$); **B.** Individual tumor sizes (mm^2) at the time of sacrifice on day 27 post-tumor injection.

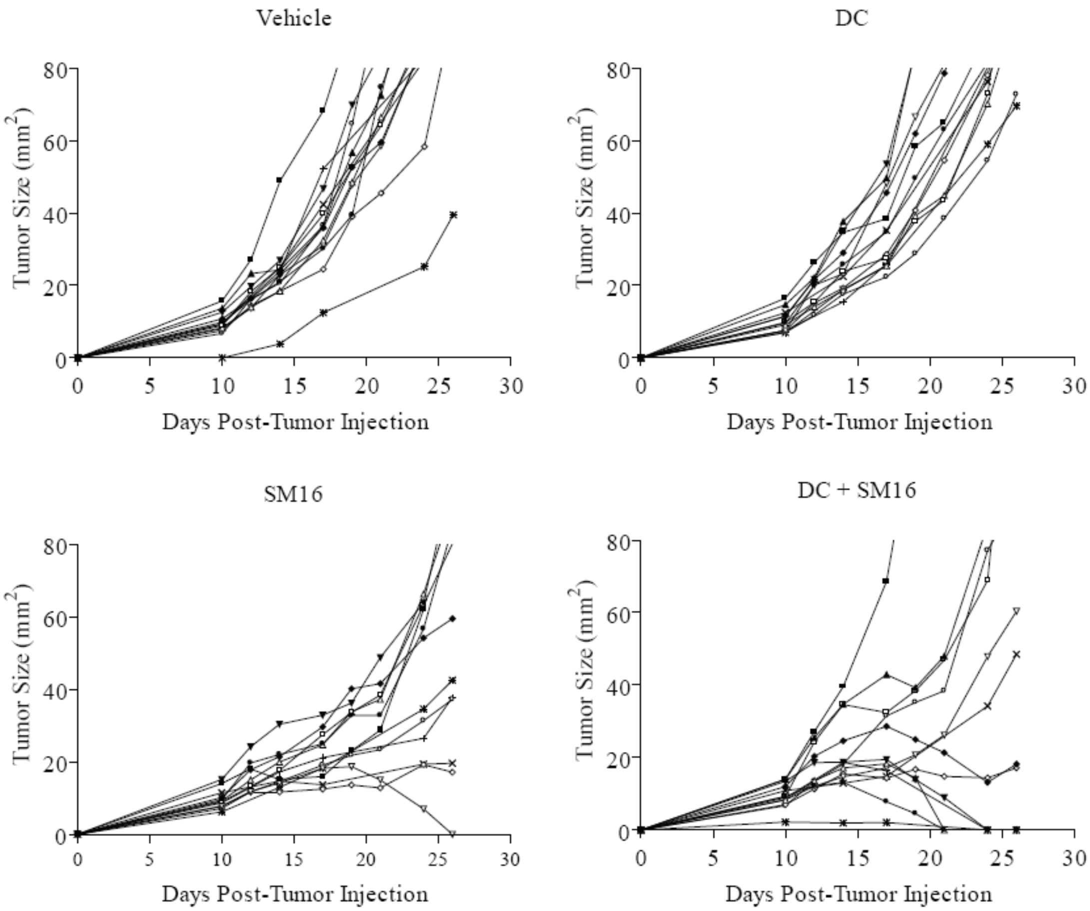


Figure 14. Effect of DC+SM16 treatment on primary tumor growth in individual mice. BALB/c mice bearing ~9-10mm^2 established 4T1 tumors were fed chow formulated with 0.45g SM16/kg chow. Mice in the DC groups were injected with 1×10^6 4T1 lysate-pulsed, TNF-α matured DCs on days 12, 17, and 22 post-tumor injection. Primary tumors were measured. The data represent individual tumor sizes (mm^2).

Table 1: DC+SM16 therapy increases the frequency of complete primary tumor regression.

Treatment Group	Number of Responders	Number of Complete Responders
Control	0/13	N/A
DC Alone	0/13	N/A
SM16 Alone	6/12 (50%)	1/6 (16.6%)
DC+SM16	7/13 (53.8%)	5/7 (71.4%)

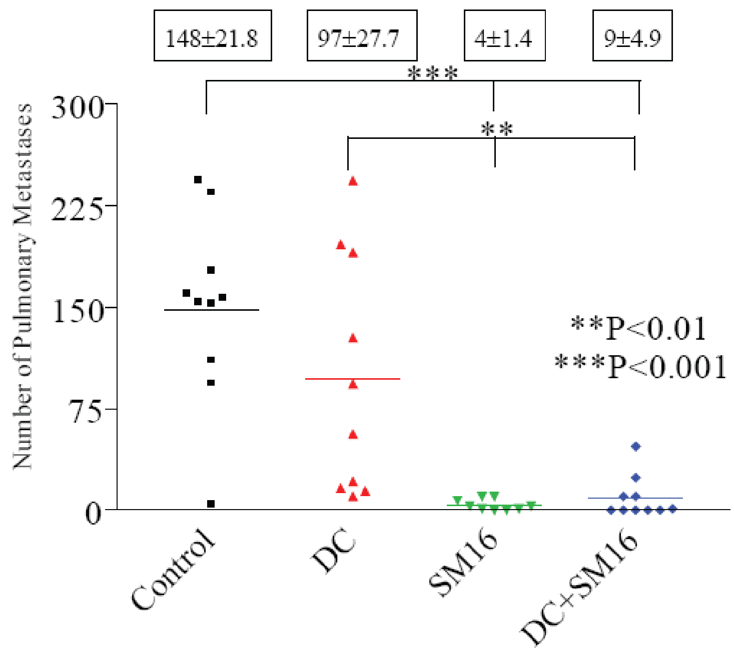


Figure 15. Effect of SM16 in combination with DC vaccination on pulmonary metastasis formation. Lung metastases were counted in tumor-bearing mice treated with oral SM16 in combination with 4T1 lysate-pulsed, TNF-α matured DCs. The data represent number of pulmonary nodules at the time of sacrifice on day 27 post-tumor injection (boxed numbers represent mean±SEM).

Aim #3: Evaluate the role of immune effector cells in the anti-tumor response following combination therapy with SM16 and DC vaccines

Inhibition of TGF-β signaling with oral SM16 enhances anti-tumor immune activity

In order to analyze the effect of DC+*p.o.* SM16 therapy on anti-tumor immune responses, splenocytes from animals in the study described above were collected at the time of sacrifice (day 27), re-stimulated with 4T1 lysate *in vitro* and analyzed for tumor-specific cytotoxicity and IFN-γ production. The data indicate that the cytolytic activity of splenocytes from mice on the SM16 diet was significantly higher ($p<0.05$) than that of cells taken from controls (Figure 16A). Furthermore, the cytolytic activity of splenocytes taken from animals treated with the combination therapy was significantly enhanced ($p<0.01$) compared to cells from all three control groups (Figure 16A). This enhanced systemic CTL activity was specific for the 4T1 mammary tumor as these effector cells had only minimal cytolytic activity against irrelevant 12B1 lymphoma tumor cells (Figure 16B). In addition, as shown in Figure 16C, splenocytes from mice in the combination DC+SM16 group produced significantly more ($p<0.001$) IFN-γ (1705.0±56.5 pg/ml) than those from mice treated with SM16 alone (122.5±4.8 pg/ml), DC alone (76.7±2.5 pg/ml), or controls (18.5±0.7 pg/ml).

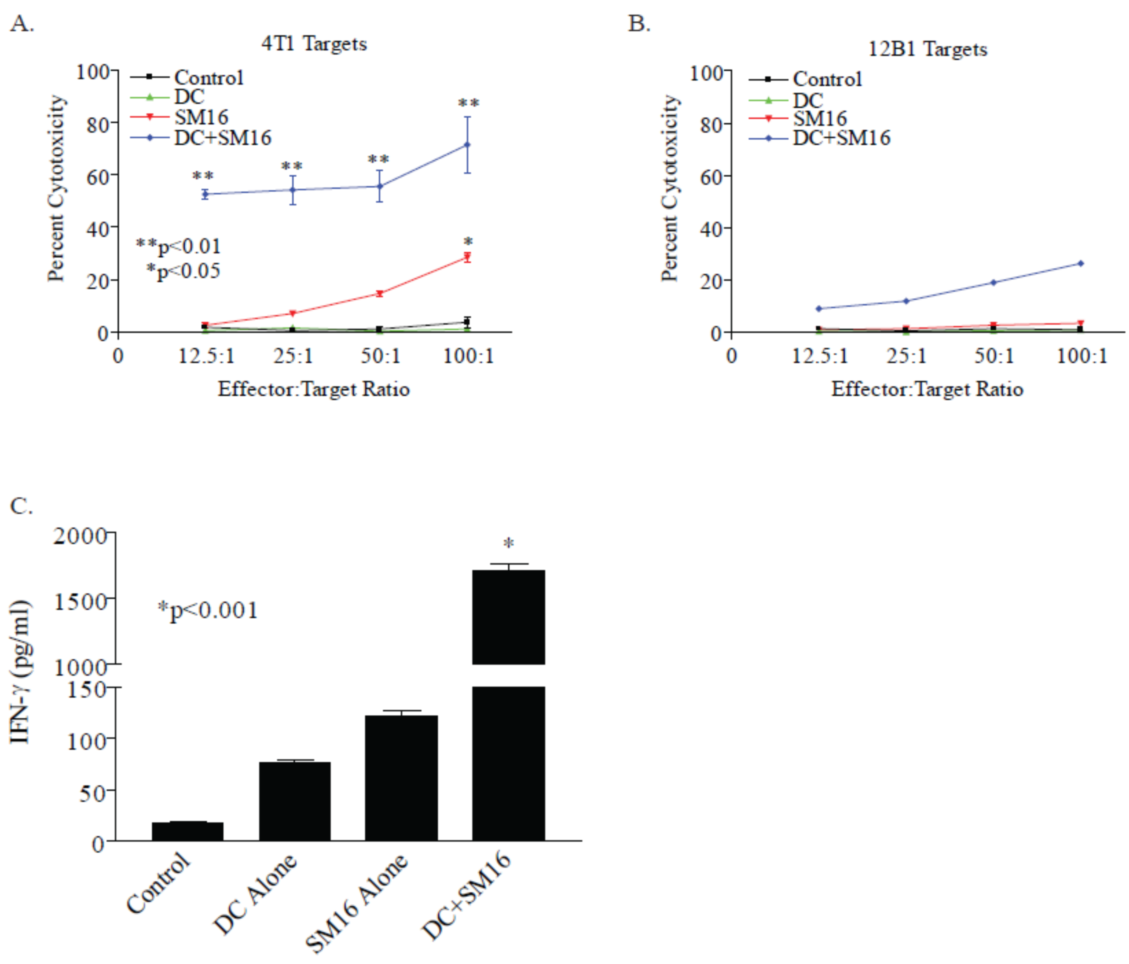
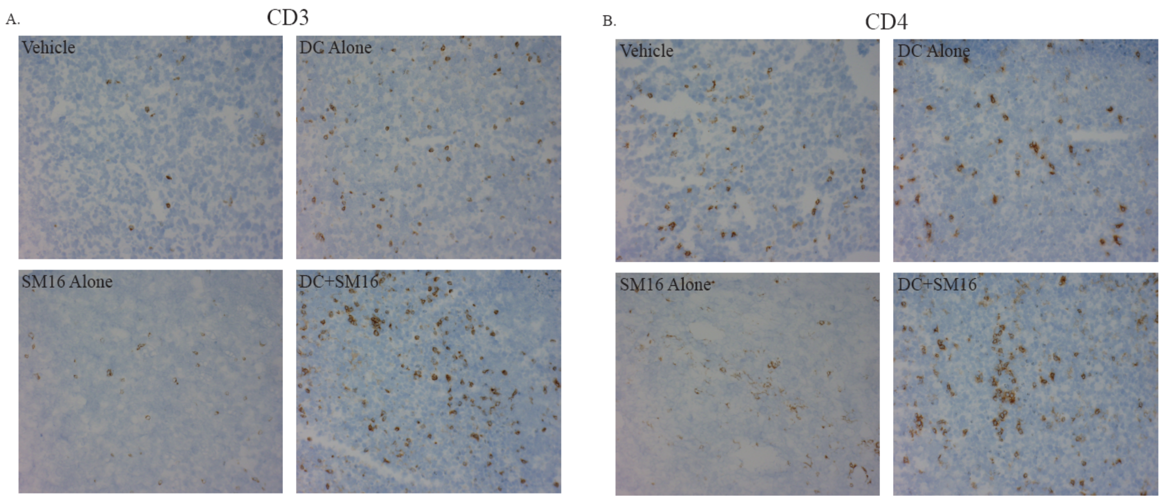


Figure 16. Effect of combination DC+SM16 therapy on anti-tumor T cell activity. A. Pooled splenocytes from treated animals (3/group) were re-stimulated *in vitro* for 6 days with 4T1 tumor lysate. Effector cells were mixed with ^{51}Cr-labeled 4T1 or 12B1 targets for 6 hr. and ^{51}Cr release

was measured. Data represent mean ± SEM of specific lysis; **B.** Splenocytes pooled from treated mice (3/group) were re-stimulated *in vitro* for 6 days with 4T1 tumor lysate and IFN-γ production was assayed by ELISA. Data represent mean ± SEM of triplicate samples.

Combination DC+*p.o.* SM16 therapy enhances T cell infiltration of primary tumors

To further assess the anti-tumor immune response generated by combination DC+*p.o.* SM16 therapy, tumors from animals in the combination therapy study described above were also collected and analyzed by immunohistochemistry for the presence of infiltrating immune cells. The data show a marked increase in $CD3^+$ and $CD4^+$ cells (>50 positive cells per high power field (HPF)) in tumors from the combination group compared to tumors from the control (~20 positive cells per HPF), DC alone (~25 positive cells per HPF), and SM16 alone (~25 positive cells per HPF) groups (Figure 17A and B). $CD8^+$ cells were rare in tumors from control animals with slightly increased numbers of $CD8^+$ cells observed in tumors from the DC alone, SM16, and DC+SM16 mice (Figure 17C). No infiltrating NK cells were observed in tumor sections from any of the treatment groups. Together with the *in vitro* T cell functional data described above, these findings demonstrate that SM16 enhances the ability of DC vaccines to stimulate anti-tumor immune activity.



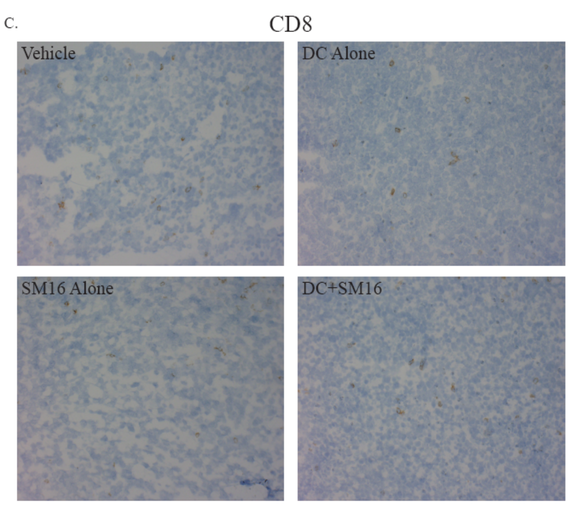


Figure 17. Effect of combination DC+SM16 therapy on primary tumor immune cell infiltration A. Tumors were removed from animals in all groups, sectioned, and stained with antibodies directed against CD3 **(A)**, CD4 **(B)**, and CD8 **(C)** antigens. Data are representative of 3 mice/group.

Year 3 (July 1st 2008 – December 12th 2008):

Aim #2: Evaluate the effect of the combination of SM16 and DC vaccination on the treatment of primary and metastatic breast *in vivo*

A suboptimal dose of oral SM16 augments the efficacy of DC cancer vaccination

Since *p.o.* SM16 at a dose of 0.45 g/kg food significantly inhibited the growth of primary and metastatic 4T1 tumors when given alone, we wanted to determine if a suboptimal dose of SM16 that lacked efficacy could enhance the effectiveness of DC vaccines. In order to find a suboptimal dose to use for our combination study, mice bearing ~9mm^2 established 4T1 tumors were fed diets with SM16 at various doses (0.7, 0.15, and 0.3 mg SM16/g food) and primary tumor growth and metastasis formation was assessed. Data from this preliminary dose titration experiment indicated that all of these doses lacked efficacy against primary and metastatic 4T1 tumors (data not shown). Therefore, we assessed the anti-tumor activity of *p.o.* SM16 at doses of 0.15 and 0.3 g/kg food in combination with DC vaccination. To this end, mice bearing ~9mm^2 established tumors were vaccinated with 4T1 tumor lysate-pulsed, TNF-α matured DC as described above in the absence or presence of oral SM16 at doses of 0.15 or 0.3 g SM16/kg chow. The data show that as expected, SM16 alone at doses of either 0.3 or 0.15 g/kg food had no effect on primary tumor growth compared to untreated controls (Figure 18A and B). Furthermore, the combination of DC+*p.o.* SM16 (0.15g/kg food) had no impact on primary tumor growth (Figure 18A). However, *p.o.* SM16 (0.3g/kg food) in combination with DC vaccination acted synergistically to significantly ($p<0.01$) delay the growth of primary 4T1 tumors relative to all other treatment groups (Figure 18B). Remarkably, two of ten animals in this combination therapy group experienced complete tumor regression. At the time of sacrifice, the combination of DC+*p.o.* SM16 (0.3g/kg food) induced a 2.4 fold reduction in primary tumor size compared to untreated controls. This relative reduction in primary tumor size is nearly identical to that induced by *p.o.* SM16 at a dose of 0.45 g/kg food (Figure 11). In terms of metastatic disease, oral SM16 at a dose of 0.15 g/kg food alone or in combination with DCs had no impact on lung metastasis (Figure 18C). However, animals treated with SM16 at a dose of 0.3 g/kg food alone or in combination with DCs had significantly fewer ($p<0.01$) pulmonary 4T1 nodules than controls (Figure 18D). Combination DC+*p.o.* SM16 (0.3g/kg food) therapy resulted in slightly reduced numbers of lung metastases compared to *p.o.* SM16 (0.3g/kg food) therapy alone, but this difference was not statistically significant.

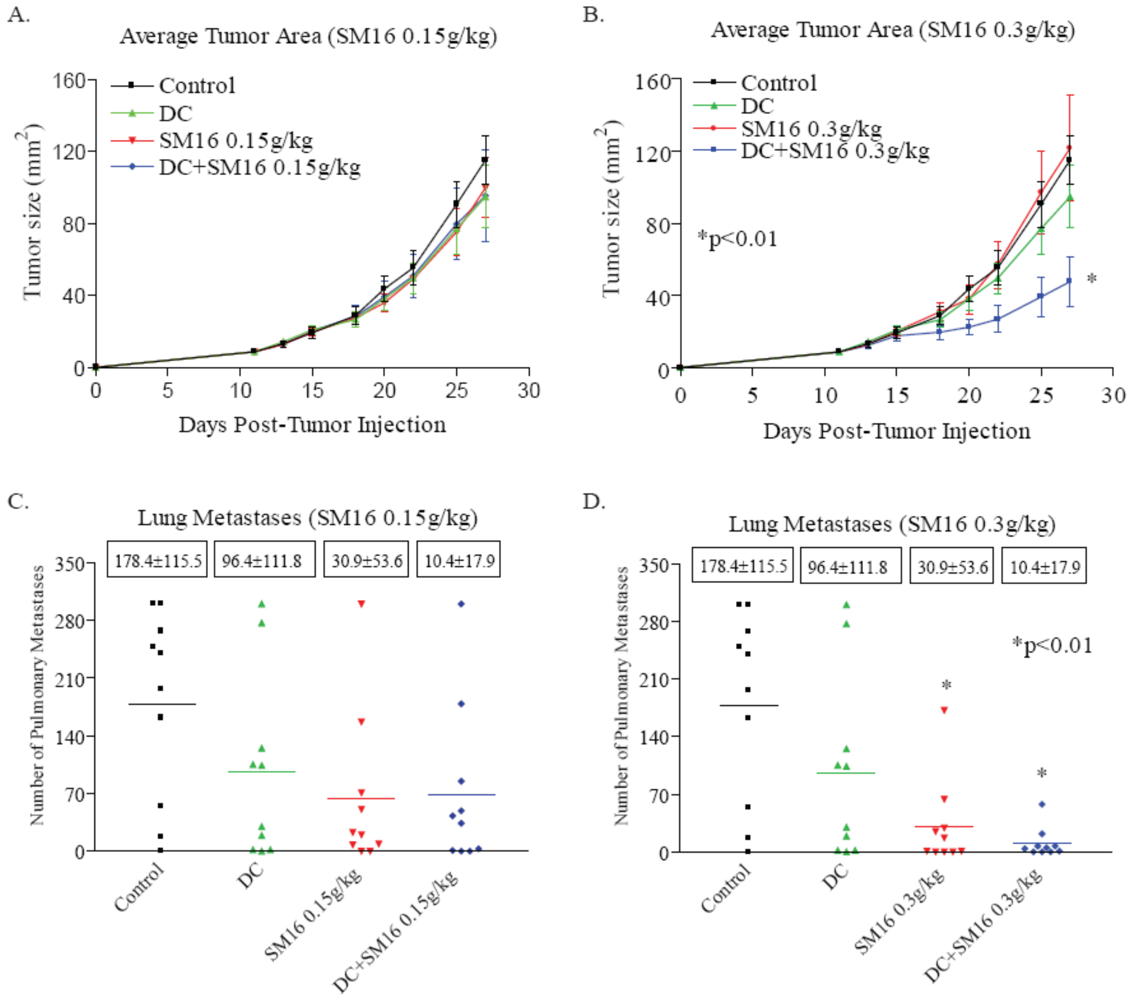


Figure 18. A suboptimal dose of oral SM16 enhances the efficacy of DC vaccines for established 4T1 tumors. BALB/c mice bearing ~9mm^2 4T1 tumors were fed to chow formulated with either 0.15 or 0.3 g SM16/kg chow. Mice in the DC groups were injected with 1x10^6 4T1 lysate-pulsed, TNF-α matured DCs on days 12, 17, and 22 post-tumor injection. Control animals received control chow alone, SM16 chow alone, or DCs alone. Primary tumors were measured and the lung metastases were counted visually. The data represent: **A and B.** Average tumor size (mm^2±SEM); **C and D.** Number of pulmonary nodules at the time of sacrifice. (boxed numbers represent mean±SEM).

Conclusions

In these studies we used two different small molecule ALK5 kinase inhibitors, HTS466284 and SM16, that interfere with TGF-β signaling in combination with DC vaccination to treat a highly metastatic, TGF-β-producing murine mammary tumor. We hypothesized that the ALK5 kinase inhibitors would render DCs resistant to TGF-β-mediated immunosuppression allowing them to stimulate more effective anti-tumor immunity. Small molecule ALK5 kinase inhibitors offer a number of advantages over other TGF-β antagonists, including superior ease of delivery, biodistribution, and ability to cross the blood brain barrier (8). While previous studies have demonstrated the potential of small molecule ALK5 kinase inhibitors to treat several murine models of cancer through immune-mediated mechanisms (2,4,5), the ability of these drugs to augment active immunization has not been addressed. To our knowledge, this is the first study to evaluate the ability of small molecule ALK5 kinase inhibitors to enhance the efficacy of adoptively transferred DC vaccines in the treatment of established tumors.

Our preliminary studies demonstrated that HTS466284 could block TGF-β signaling events in cultured 4T1 tumor cells and established 4T1 tumors and that this drug could restore the immunostimulatory capacity of TGF-β-treated DCs *in vitro.* However, despite the ability of HTS466284 to block some of the tumor promoting effects of TGF-β *in vitro*, it was ineffective against established 4T1 tumors *in vivo* at the maximum tolerated dose of 45mg/kg body weight. Treatment of tumor-bearing mice with HTS466284 at this dose failed to control the growth and metastasis of established 4T1 tumors. The failure of HTS466284 to inhibit the formation of 4T1 pulmonary metastases is particularly interesting given that this drug significantly blocked TGF-β–induced EMT and migration *in vitro* in preliminary studies.

HTS466284 also failed to improve the efficacy of DC vaccination against established and metastatic 4T1 tumors. The failure of HTS466284 to consistently control tumor growth *in vivo* suggests that HTS466284 may not be an effective ALK5 kinase inhibitor for use in cancer therapy. Several explanations may account for this lack of efficacy. First, the duration of TGF-β signal inhibition from a single *i.p.* dose of HTS466284 may be limited. While we show that one injection of HTS466284 causes a near complete inhibition of Smad2 phosphorylation in primary tumor tissue 1 hr. after therapy, we do not know the duration of this signal blockade. Additional experiments are necessary to analyze the time course of TGF-β signal inhibition in 4T1 tumors from a single dose of HTS466284. Second, the solubility of HTS466284 is limited to organic solvents such as DMSO which can be toxic in animals when used as a delivery vehicle. Third, the dose-limiting toxicity of HTS466284 prevented us from investigating doses above 45 mg/kg.

Since HTS466284 lacked activity against 4T1 tumors in our initial studies, we shifted our focus to SM16 which was developed by optimizing HTS466284 for enhanced biological activity (1). As a result, SM16 possesses a number of properties, such as solubility in aqueous solvents and oral bioavailability, that make it better suited for *in vivo* dosing. Similar to what was observed with HTS466284, SM16 inhibited Smad2 phosphorylation in both cultured 4T1 cells and primary and metastatic 4T1 tumor tissue. Blockade of TGF-β signaling with SM16 also inhibited 4T1 cell invasion *in vitro*. Taken together, these findings show that SM16 is capable of blocking several tumor promoting effects of TGF-β in the 4T1 tumor model.

Having shown that SM16 can effectively inhibit TGF-β signaling *in vitro*, we next wanted to test its *in vivo* efficacy. Our data shows that when delivered via daily i.p. injection, SM16 has no effect on the growth of established primary tumors, but does inhibit the growth of pulmonary metastases at a concentration of 40mg/kg. SM16 when delivered i.p. was less effective at treating larger established primary tumors, but still led to a marked reduction in the incidence of

spontaneous pulmonary metastases in these mice. This observation suggests that i.p.SM16 therapy may be more effective at treating disseminated disease, where tumor burden is minimal and is in agreement with previous reports by us (3) and others (1,4,5,7) that the impact of TGF-β inhibition on metastasis formation is superior to its effect on established primary tumors.

Having shown that i.p. delivery of SM16 resulted in suppression of metastasis formation, we decided to test the efficacy of orally delivered SM16. Suzuki et al. have previously demonstrated that oral administration of SM16 was safe and highly effective in a murine mesothelioma model (2). Our data indicate that oral administration of SM16 was safe and resulted in a rapid accumulation of SM16 in the serum that was maintained for the duration of the study. The suppression of primary tumor growth and metastasis of established 4T1 tumors by orally delivered SM16 was enhanced compared to i.p. administration suggesting continuous dosing may result in more sustained and complete inhibition of TGF-β signaling which may be more desirable for controlling established primary tumors.

Furthermore, we show that the anti-tumor activity of orally delivered SM16 is dependent on an immune component. The loss of efficacy of SM16 in immunodeficient SCID mice suggests SM16's mechanism of action requires adaptive cellular immunity. Although we demonstrate in this study that SM16 blocks invasion of 4T1 tumor cells *in vitro*, this mechanism does not appear to play a major role in the anti-metastatic effects of this drug *in vivo* as SM16 had no effect on the formation of lung metastases in immunodeficient SCID mice. This finding is consistent with that of Ge *et al.* (5), who showed that SD-208 had no effect on the metastasis of R3T mammary tumors in athymic nude mice despite its ability to inhibit R3T invasion *in vitro*. However, these findings are in contrast to a report by Bandyopadhyay *et al.*(7) showing that a small molecule ALK5 kinase inhibitor reduced the ability of human breast tumor (MDA-MB-435) xenografts to form spontaneous pulmonary metastases by blocking the tumor cell autonomous effects of TGF-β. The disparity in these findings may be attributed to the different tumor models (mouse versus human) used in both studies.

In this study we also investigated the efficacy of SM16 in combination with DC vaccination in the treatment of established 4T1 tumors. Our data indicate that the antitumor effect of DC+i.p.SM16 did not differ from that of i.p. SM16 alone. Similarly, there was no difference in average primary tumor growth between mice receiving *p.o.* SM16 alone and those recieving DC+*p.o.* SM16. In our oral delivery experiments, both the SM16 alone and DC+SM16 groups had similar numbers of animals that responded to therapy. However among the responders, *p.o.* SM16 plus DC vaccination resulted in an increased frequency of primary tumor regression than *p.o.* SM16 alone. This data suggests that continuous SM16 dosing which is achieved through dietary administration may be required to produce sustained inhibition of TGF-β-induced immunosuppression to allow for the generation of effective anti-tumor immunity by our DC vaccine.

The fact that some animals responded to DC+ *p.o.* SM16 and some animals did not suggests that heterogeneity exists in the tumors in these animals. One possible explanation for this observation is that tumors that grew progressively in mice that did not respond to combination DC+SM16 therapy may have lost the expression of one or more immunodominant antigens necessary for immune-mediated destruction. In order to determine if heterogeneity due to antigen loss exists in these tumors, transplantation experiments could be performed in which tumors from nonresponder animals are transplanted into responder animals and vice versa and tumor growth could be followed.

In this study the superior anti-tumor efficacy of DC+p.o.SM16 correlated with enhanced IFN-γ production and 4T1-specific cytolytic activity by splenocytes and increased T cell infiltration of primary tumors. This enhanced T cell-mediated anti-tumor activity recapitulates the anti-tumor responses seen when TGF-β signaling is inhibited genetically in DC or T cells through the expression of a dominant negative TβRII transgene (9-10). However, pharmacological inhibition of TGF-β signaling with orally administered SM16 appears to be a safer and less complex approach that does not result in the fatal autoimmune inflammatory disease seen when TGF-β signaling is inhibited genetically in T cells (11).

While our data demonstrate that T cell immunity clearly plays a role in anti-tumor mechanism of DC+ *p.o.*SM16 therapy, our work does not definitively identify the specific T cell subsets involved in this response. In order to address this question, these experiments could be repeated in mice depleted of either $CD4^+$, $CD8^+$, or both $CD4^+$ and $CD8^+$ T cells prior to therapy. In addition, the presence of $CD4^+CD25^+FoxP3^+$ T cells could be analyzed in lymphoid tissue from treated animals to determine if SM16 therapy impacts regulatory T cell numbers.

Taken together, our data indicate that SM16 is a safe and highly effective drug for the treatment of metastatic breast cancer. However, given the critical importance of TGF-β in a number of normal cellular processes (12), it is likely that dose limiting toxicities may emerge that will ultimately impede the use of these agents in the clinic. Our data indicate that a lower dose of SM16 (0.3 g SM16/kg chow) that is non-toxic and does not display therapeutic efficacy against established tumors on its own can also augment the anti-tumor activity of DC vaccination. Therefore, even if side effects limit the use of ALK5 kinase inhibitors to suboptimal doses in future human trials, combination strategies with DC-based immunotherapy may offer one potentially useful approach to improve efficacy.

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